Evaluation of Styrene Mouse Cancer Studies
February 2009

Summary

The National Toxicology Program (NTP) Report on Carcinogens (RoC) criteria for listing substances as reasonably anticipated to be human carcinogens based on animal data requires two studies of clear evidence of increased cancer in two or more species or by two or more routes. For styrene, the RoC Draft Substance profile asserts that styrene caused a clear increase in lung cancer in both sexes in a mouse inhalation study (Cruzan et al., 2001) and in male mice by gavage in which there were 20 male and 20 female control mice and 50 male and 50 female in each of the two treated groups (NCI, 1979a).

There are four gavage studies of styrene which provide conflicting data and no more than suggestive evidence of carcinogenicity. The results are summarized below:

Chemical/Species	Doses (mg/kg)	Results	Reference
Styrene in B6C3F1	0, 150, 300	Increase in high dose males within historical control range NCI: Suggestive evidence	NCI, 1979a
Styrene/β-nitrostyrene in B6C3F1	0, 204, 408	Increase in low dose males NCI: no convincing evidence	NCI, 1979b
Styrene in O20	0, 1350	Increased lung tumors male and female mice. Severe lung toxicity with 50% mortality by week 20.	Ponomarkov, 1978
Styrene in C57Bl	0, 300	No increase in lung tumors	Ponomarkov, 1978

The NTP concluded that the NCI (1979a) study of styrene provided clear evidence of increased lung tumors because the styrene incidences were different from a new historical control they developed, using corn oil controls from the Litton laboratory where the study was conducted (2 studies) and 12 studies from nearby Hazleton laboratory. Haseman et al. (1985) concluded that the use of corn oil did not impact tumor incidence in B6C3F1 mice in NCI-NTP carcinogenesis bioassays. These investigators also observed that the incidence of mouse lung tumors exhibited significant inter-laboratory variability and recommended that use of historical control tumor incidence values to facilitate bioassay interpretation should be restricted to values developed within the testing laboratory (Haseman et.al. 1984). A more thorough evaluation of NCI bioassays at Litton and Hazleton at the time of the styrene study revealed that the lung tumor incidence at Hazleton (28 studies by corn oil gavage or diet) was 3% while that at Litton (13 studies by corn oil gavage or diet) it was 9% for studies of approximately 91 weeks. A laboratory difference in lung tumor incidence was further supported by examining 104-week studies at the two labs. The incidence at Hazleton was 11% (7 studies, range 2-18%) and at Litton was 19% (40 studies, range 0-45%).

Thus the new historical control presented by NTP (2008) is not valid for assessing the styrene study. The incidence of lung tumors in the high dose of the styrene study (TR185) was less than the control incidence of 2 of the 15 Litton studies. The original conclusion of the NCI should be retained; the study is no more than suggestive evidence of carcinogenicity of styrene.

There is only clear evidence of styrene-induced lung tumors in mice by inhalation (Cruzan et al., 2001), and no more than suggestive evidence by gavage in mice. These data together constitute limited evidence in animals, not sufficient evidence.

In addition, more recent evaluations of the lung tumors observed in mice following inhalation exposure to styrene suggest that they are caused by a non-genotoxic mode of action that is unique to mouse metabolism and quantitatively different from humans (Cruzan et.al., 2002). As such, these results are of questionable value to human hazard or risk estimation. The NTP Draft Substance Profile fails to adequately discuss this mode of action which is supported by a robust scientific literature and instead focuses almost entirely on a hypothesized alternative genotoxic mode of action.

Mouse Studies

NCI (1979a) administered styrene in corn oil by gavage to 20 male and 20 female control mice and 50 male and 50 female in each of the two treated groups, 150 and 300 mg/kg day (NCI, 1979a). There was an increased trend for lung tumors in males and the high dose was significantly elevated compared to the concurrent control (0, 12 and 18% at 0, 150 and 300 mg/kg/day styrene in corn oil). NCI concluded that the evidence was no more than suggestive. The report identifies 2 studies of chemicals dissolved in corn oil at the same laboratory, including the styrene study. They concluded that this was insufficient to assess the historical control range and relied on the incidence in control mice from several diet studies performed at the same laboratory at about the same time. The historical control incidence of the studies they selected averaged 12% with a range up to 20%. The primary reviewer of the styrene report recommended that this study provided suggestive evidence of carcinogenicity, while the secondary reviewer recommended that the study was negative. The NCI concluded that the styrene incidence was within the historical range and the styrene difference from control provided no more than suggestive evidence.

The NTP (2008) reasoned that using untreated mice as the historical control was incorrect, so they developed a new historical control using corn oil controls from the two studies at Litton plus 12 additional studies at nearby Hazleton Laboratories from the first 110 TR numbers. The Hazleton studies included nearly all NCI studies done at Hazleton with a lower TR number than the styrene study (TR185). This control had an average incidence of lung tumors of 4%. Therefore, the NTP report concluded that the control incidence in the Litton styrene study was not low, the incidence in the styrene study was outside the historical control range, and the NCI study provided clear evidence of carcinogenicity of styrene by oral gavage.

The NTP failed to assess whether there was a difference in lung tumor rates in male B6C3F1 mice at Litton and at Hazleton. The NCI used a group of diet studies from Litton as comparison, with an average incidence of 12% and a range to 20%. NTP compared the styrene study to the incidence in 14 control groups administered corn oil from Litton (2) and Hazleton (12). We examined not only all the corn oil control studies at Hazleton, but also all the diet studies at Hazleton conducted about the same time as the styrene study. We found two additional corn oil studies not included in the NTP database (total 14) and 14 diet studies of approximately 91 weeks. The average incidence (and range) for the corn oil studies was 4% based on 10/256 male mice (range 0-18%) and for diet studies was 2% based on 6/260 male mice (0-11%). These data confirm the conclusion of Haseman et al. (1985) that the use of corn oil has little impact on the incidence of tumors in the NCI-NTP carcinogenicity studies, and particularly so in B6C3F1 mice.

The NCI styrene study (TR185) included a historical control for male mouse lung tumors of 12% in diet studies at Litton around the time of the styrene study; unfortunately, the report does not indicate which studies were included. We examined the NTP database for diet studies with a TR number less than 190 and duration of approximately 92 weeks. We found 14 such studies; the average incidence was 11% (29 /271) with a range of 0-20%. Furthermore at Litton, 2 of the 14 diet studies had a control incidence that was greater than the incidence in the styrene high dose males (18%). Similarly, the 104-week studies at Hazleton had a lower lung tumor incidence (11%) than the 104-week studies at Litton (19%).

Three conclusions can be drawn from this analysis:
1. The overall incidence of alveolar/bronchiolar adenomas or carcinomas in male control mice at Litton in studies TR000-TR190 of ~91 weeks duration was 9.1%. The incidence in the high dose styrene exposed males (18%) was lower than the incidence in the control males of 2 of 15 Litton studies during this period.

2. The incidence of alveolar/bronchiolar adenomas or carcinomas in male mice at Litton was much greater than at Hazleton (overall 9.1% vs, 3.1% for 91 week studies; 19 vs 11% for 104 week studies). Therefore, the control data at Hazleton is not a valid comparison for Litton studies.

3. The NCI conclusion that TR185 provides suggestive evidence is the correct interpretation of the study and the NTP conclusion of clear evidence is not valid.

The scientific justification presented in the Draft Substance Profile for use of corn oil historical controls from a different testing laboratory than the one in which the styrene oral gavage study (TR185) was conducted is not supported by any peer-reviewed scientific analyses. In fact, published analyses by NTP investigators have concluded: 1) statistically significant inter-laboratory variability in control mouse lung tumor incidence indicates that intra-laboratory controls should be used for historical control comparisons (Haseman et.al., 1984; and 2) the incidence of historical control mouse lung tumors in the NCI/NTP database is not affected by corn-oil vehicle treatment (Haseman et.al., 1985).

Other Studies in Animals

The NCI also conducted a gavage study of commercial β-nitrostyrene (TR170) in B6C3F1 mice. Commercial β-nitrostyrene is 30% β-nitrostyrene and 70% styrene. Therefore, the mice that received β-nitrostyrene received 2.3 times as much styrene. The β-nitrostyrene doses of were 87.5 and 175 mg/kg 3 days per week for 78 weeks, followed by 14 weeks of observation. The styrene doses were 204 and 408 mg/kg/day 3 days per week. Note that the daily doses of styrene were greater than those of styrene by itself (TR185). Again the incidence of alveolar/bronchiolar adenomas or carcinomas in the control male mice was 0 of 20. At the lowest dose, 11 of 50 male mice dosed at 204 mg/kg/day styrene developed lung tumors, while only 2 of 36 males dosed at the highest dose of 408 mg/kg had lung tumors. Fourteen male high-dose mice died during week 40, which was attributed to a handling accident. The average weekly doses of styrene in TR170 and TR185 were: low dose - 87.5 vs. 107 mg/kg/day averaged over 7 days/week, and high dose – 175 vs. 214 mg/kg/day averaged over 7 days/week. (Calculated by daily dose x days/week dosed/ 7 days/week.) Thus at similar doses, styrene (dosed with β-nitrostyrene) did not increase lung tumors in male mice.

In a study of unusual design (Ponomarkov and Tomatis, 1978), styrene (in olive oil) was administered to 29 pregnant female O20 mice on gestation day 17 at 1350 mg/kg. Surviving offspring were administered styrene in olive oil once per week beginning at weaning (21 days of age) at 1350 mg/kg. Preweaning mortality among the control mice was 22%, indicating a less than healthy population. Preweaning mortality among offspring of the dams treated with styrene during gestation was 43%, indicating not only a less than healthy population, but also severe toxicity from styrene. Nevertheless, the investigators initiated dosing at 1350 mg/kg, which continued for 16 weeks. There was so much toxicity that 50% of the styrene-treated male mice had died by week 20. Pathology indicated necrosis of the liver, hypoplasia of the spleen and severe congestion of the lungs. Male mice had a significantly higher incidence of lung tumors than the olive oil controls, but not significantly different from untreated controls. In females, the styrene treated mice had significantly greater incidence of lung tumors than either control group. Early lung tumors in the presence of severe lung toxicity should not be surprising. Styrene has been shown to cause decreases in the level of CC10. Decreased CC10 leads to increased lung tumors in mice. Hicks et al. (2003) showed that nearly all CC10-KO mice had hyperplasia in the terminal bronchioles by 1 month of age and lung adenomas by 2 months of age. CC10 was not detectable in whole lung homogenates at any time from these transgenic mice.

Ponomarkov and Tomatis (1978) also administered styrene using the same protocol to C57Bl mice at 300 mg/kg. No lung toxicity was seen and there was no increased in lung tumors.

Conclusion

Based on these data (summarized in Attachment A), it appears that the NTP analysis performed on the NCI mouse study (TR185) is inappropriate and does not support the

conclusion of sufficient evidence of increased cancer incidence in experimental animals. Based on the NCI conclusion of suggestive evidence in TR185, no evidence in TR170, no increase in C57Bl mice, and increased lung tumors in the presence of severe lung toxicity in O20 mice, the evidence by oral administration of styrene provides no more than suggestive evidence of increased lung tumors. The NTP criteria for sufficient evidence in animals requires increased cancer by two routes of administration (if found in only one species). There are increased lung tumors from inhalation exposure of styrene in mice, but only suggestive evidence by oral gavage. This does not meet the NTP standard of "sufficient evidence."

References

Cruzan G, Cushman JR, Andrews LS, Granville GC, Johnson KA, Bevan C, Hardy CJ, Coombs DW, Mullins PA, Brown WR. 2001. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. *J Appl Toxicol* 21(3): 185-198.

Haseman JK, Huff, J, Boorman, GA. 1984. Use of historical control data in carcinogenicity studies in rodents. *Toxicologic Pathology* 12: 126-135

Haseman JK, Huff JE, Rao GN, Arnold JE, Boorman GA, McConnell EE. 1985. Neoplasms observed in untreated and corn oil gavage control groups of F344 rats and (C57Bl/6N x C3H/HeN)F1 (B6C3F1) mice. JNCI. 75: 975-984.

Hicks SM, Vassallo JD, Dieter MZ, Lewis CL, Whiteley LO, Fix AS; Lehman-McKeeman LD. 2003. Immunohistochemical analysis of Clara cell secretory protein expression in a transgenic model of mouse lung carcinogenesis. Toxicology 187:217-228.

NCI. 1979a. *Bioassay of Styrene for Possible Carcinogenicity*. Technical Report Series No. 185. Bethesda, MD: National Cancer Institute.

NCI. 1979b. *Bioassay of a Solution of β-Nitrostyene and Styrene for Possible Carcinogenicity*. Technical Report Series No. 170. Bethesda, MD: National Cancer Institute.

Ponomarkov V, Tomatis L. 1978. Effects of long-term oral administration of styrene to mice and rats. *Scand J Work Environ Health* 4(Suppl 2): 127-135.

Attachment A: Control Data from NCI Bioassays Performed at Litton or Hazleton from Study Numbers TR000-TR219

	No. of studies	No. of lung tumors	No. of mice examined	Average% of tumors	Range
Litton ~91 week studies					
Corn oil gavage	2	0	40	0	0
Untreated	14	29	271	11	0-20
Overall	16	29	311	9	0-20
Hazleton ~91 week studies					
Corn oil gavage	14	10	256	4	0-18
Untreated	14	6	260	2	0-11
Overall	28	16	516	3	0-18
Litton ~104 week studies					
Corn oil gavage	1	1	20	5	5
Water gavage	4	9	79	11	10-15
Untreated	35	157	780	20	0-45
Non-corn oil	39	166	859	19	0-45
Overall	40	167	879	19	0-45
Hazleton ~104 week studies					
Untreated	7	38	341	11	2-18